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A Spontaneously Arising Mutation in the DLAARN Motif of Murine ZAP-70 Abrogates Kinase Activity and Arrests Thymocyte Development

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Summary

Development of immature CD4$^+$CD8$^+$ thymocytes into functionally mature CD4$^+$ and CD8$^+$ T cells is driven by selection events that require signals transduced through the T cell antigen receptor (TCR). Transduction of TCR signals in the thymus involves tyrosine phosphorylation of the protein tyrosine kinase ZAP-70 by p56^{lck} and results in induction of ZAP-70 enzymatic activity. We have identified a novel, spontaneously arising point mutation within a highly conserved motif (DLAARN) in the kinase domain of murine ZAP-70 that uncouples tyrosine phosphorylation of ZAP-70 from induction of ZAP-70 kinase activity. Mice homozygous for this mutation are devoid of mature T cells because thymocyte development is arrested at the CD4$^+$CD8$^+$ stage of differentiation. The developmental arrest is due to the inability of CD4$^+$CD8$^+$ thymocytes to propagate TCR signals in the absence of ZAP-70 kinase activity despite tyrosine phosphorylation of TCR-associated ZAP-70 molecules.

Introduction

The repertoire of T cell antigen receptor (TCR) specificities expressed by mature CD4$^+$ and CD8$^+$ T cells is selected from among the wider array of immature T cell precursors in the thymus. This selection process is exerted on thymocytes at the CD4$^+$CD8$^+$ stage of differentiation in that CD4$^+$CD8$^+$ thymocytes expressing TCRs reactive with self-antigens are deleted, whereas those expressing TCRs with potential reactivity against foreign antigens are signaled to mature into CD4$^+$ or CD8$^+$ T cells (Robey and Fowlkes, 1994). These selection processes are dependent on TCR signals mediated by protein tyrosine kinase (PTK) molecules (Littman et al., 1994; Hashimoto et al., 1996), particularly the Syk-family PTK ZAP-70 (Chan et al., 1991, 1992). Indeed, thymocyte development is arrested in genetically engineered animals that do not express ZAP-70 protein (Negishi et al., 1995). While it is clear that ZAP-70 PTK molecules are necessary for thymocyte development, the precise role of ZAP-70 in this process remains unclear. One role for ZAP-70 in thymocyte development might be to propagate TCR signals by phosphorylating cytosolic proteins, such as SLP-76, that are part of the TCR signaling cascade (Wardenburg et al., 1996). Alternatively, by phosphorylation of its tyrosine residues, ZAP-70 might serve as a scaffold to which signaling molecules are recruited and assembled into signaling complexes. In fact, it has been proposed that the primary function of ZAP-70 kinase activity is to create this scaffold by autophosphorylation (Neumeister et al., 1995).

In this report, we describe a novel, spontaneously arising mutation of arginine to cysteine at position 464 (R464C) in a highly conserved motif within the kinase domain of ZAP-70 (DLAARN) (Hanks and Quinn, 1991) that abrogates ZAP-70 kinase activity, blocks signaling though the TCR complex, and arrests thymocyte development, but does so without preventing phosphorylation of ZAP-70.

Results

While inbreeding mice that were heterozygous for targeted disruption of the CD28 gene, we found a number of mice that were devoid of peripheral T lymphocytes, a phenotype we named "ST" (for "strange") (Shahinian, et al., 1993). The ST phenotype was heritable and segregated as an autosomal recessive. Indeed, all F1 progeny from ST × ST matings were phenotypically ST, whereas all F1 progeny from mating ST mice with normal C57BL/6 (B6) animals were phenotypically normal. In addition, intercrossing the normal F1 progeny of ST × B6 matings resulted in expression of the ST phenotype in 29 of 127 (approximately one fourth) of the resultant F2 progeny. Although originally identified in CD28$^{+/-}$ heterozygous mice, the ST phenotype segregated independently of CD28 expression. All ST mice described in this report were homozygous CD28$^{+/+}$ and did not contain the gene-targeting vector in their genomic DNA. We conclude that the ST phenotype resulted from a spontaneous mutation that occurred either in vivo in the CD28$^{+/-}$ heterozygous animal colony or in vitro in the embryonic stem cell line from which the CD28$^{+/-}$ mice were derived.

The peripheral lymphoid organs of ST mice lacked both CD4$^+$ and CD8$^+$ T cells but contained B220$^+$ B lymphocytes (Figure 1A), indicating that the effects of the ST mutation were limited to the T cell lineage. Thymic cellularity was significantly reduced in ST mice, and ST thymocytes failed to mature beyond the CD4$^+$CD8$^+$ stage of development (Figure 1A). The developmental arrest of ST thymocytes was not the result of defects in either TCRα or TCRβ expression, since the ST phenotype was not affected by introduction of productively

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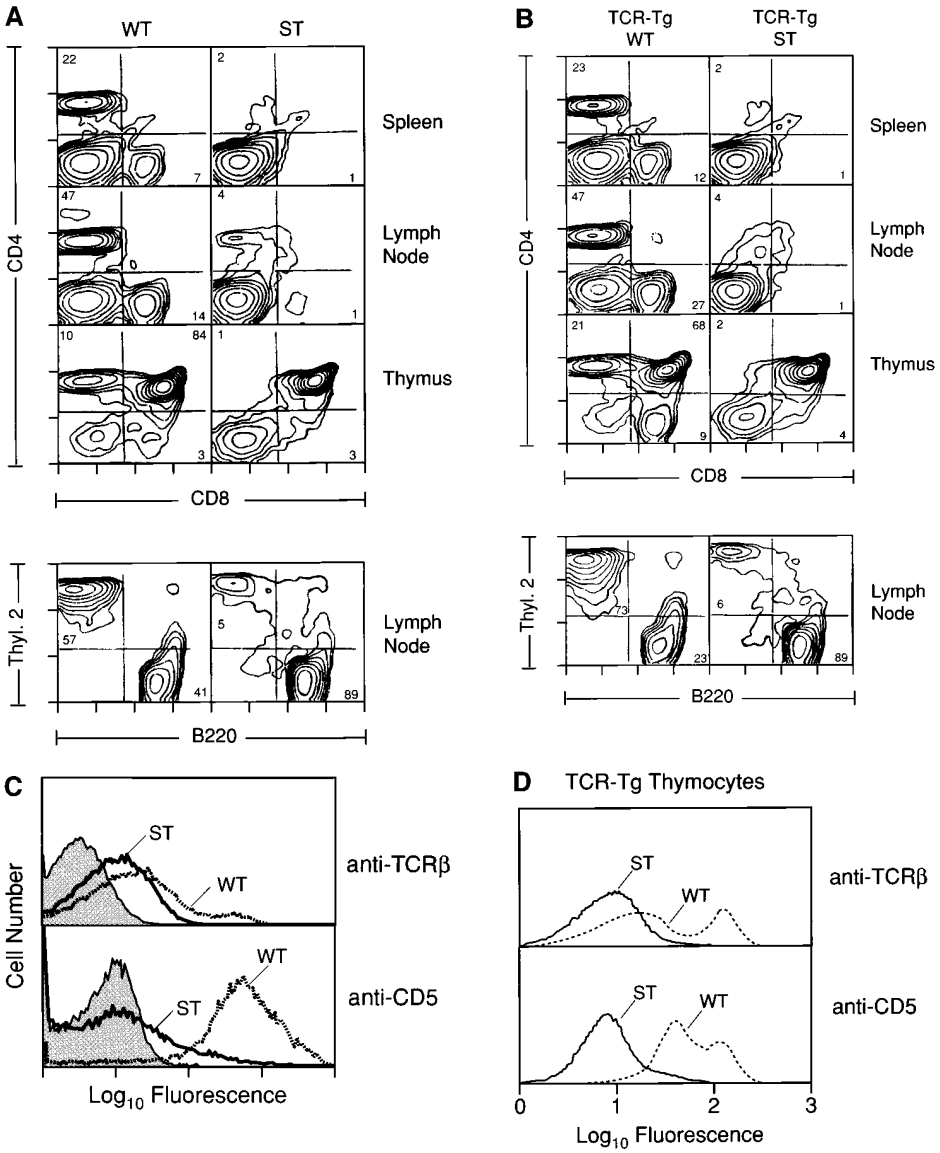

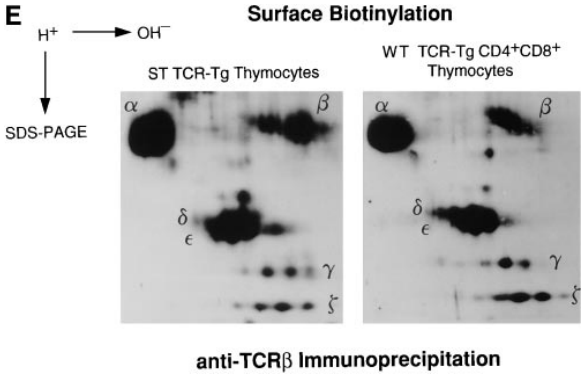

Figure 1. ST Mice Lack Peripheral T Cells Because of an Arrest in Thymocyte Development

(A and B) Spleen, lymph node, and thymus cells from WT B6 and ST mice (A) and from WT and ST mice expressing the DO.11.10 TCRαβ transgenes (B) were analyzed by immunofluorescence and multicolor flow cytometry. Cells were stained for CD4 versus CD8 expression to identify $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$ T cell subsets (top) and for Thy1.2 versus B220 expression to identify $Thy1^+$ T cells and $B220^+$ B cells (bottom). Spleen and lymph nodes of ST mice were essentially devoid of T lymphocytes but did contain normal numbers of B cells. The thymi of ST mice contained only immature $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes and were devoid of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. (C and D) TCRβ and CD5 surface expression were assessed on thymocytes from WT and ST mice (C) and on thymocytes from WT and ST mice expressing the D0.11.10 TCRαβ transgenes (TCR-Tg) (D).

(E) TCR subunits of ST thymocytes display normal electrophoretic mobility. Detergent extracts of surface biotin–labeled TCR-transgenic WT and ST thymocytes were immunoprecipitated with anti-TCRβ MAb (H57–597), and the resultant immune complexes were resolved on two-dimensional electrophoresis gels based on charge in the first dimension (nonequilibrium pH-gradient electrophoresis) and size in the second dimension (SDS-PAGE). The migration position of each TCR subunit is indicated.

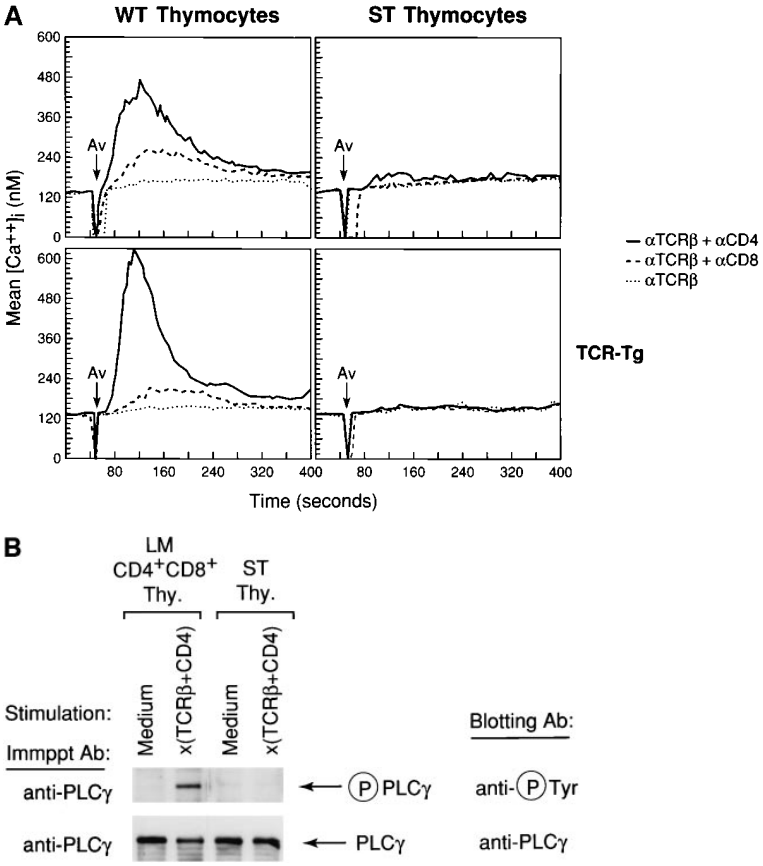

Figure 2. Defective TCR Signaling in ST Thymocytes

(A) ST thymocytes are unable to mobilize intracellular calcium in response to co-crosslinking of surface TCR and CD4. Intracellular calcium mobilization in response to TCR signaling was assessed in purified $CD4^+CD8^+$ thymocytes from WT and ST nontransgenic thymocytes (top) and in WT and ST transgenic mice expressing the DO.11.10 TCR$\alpha\beta$ transgenes (TCR-Tg) (bottom). Crosslinking of surface receptors was accomplished by addition of streptavidin to thymocytes that had been stained with biotin-labeled anti-TCRβ MAb either alone or in combination with biotin-labeled anti-CD4 or anti-CD8 MAbs.

(B) Co-crosslinking of TCR + CD4 fails to induce tyrosine phosphorylation of PLCγ in ST thymocytes. Tyrosine phosphorylation of PLCγ was measured in $CD4^+CD8^+$ thymocytes from normal littermate (LM) or ST mice after 5 min of TCR + CD4 co-crosslinking. PLCγ was isolated by immunoprecipitation with specific antibody (anti-PLCγ), resolved by SDS-PAGE, and then blotted with anti-phosphotyrosine MAb (4G10). Bound antibody was visualized using horseradish peroxidase–conjugated protein A and chemiluminescence. The same membranes were then washed and reprobed with anti-PLCγ antibody, and bound antibody was visualized by ^{125}I-protein A and autoradiography.

rearranged TCRα and TCRβ (D011.10) transgenes (Figure 1B) (Murphy et al., 1990). Surface TCR levels were reduced on ST thymocytes (Figure 1C), including ST thymocytes expressing TCR$\alpha\beta$ transgenes (Figure 1D), but reduced TCR expression was most likely a consequence rather than a cause of the developmental arrest in ST mice, since TCR expression was only marginally lower than that on the TCR^{lo} subset of normal wild-type (WT) $CD4^+CD8^+$ thymocytes. Furthermore, all subunits (α, β, γ, δ, ϵ, and ζ) of the complete TCR complex were present on ST thymocytes, and their charge and size appeared normal as revealed by migration in two-dimensional nonequilibrium pH-gradient electrophoresis (Figure 1E).

In contrast to TCR expression, surface expression of the activation marker CD5 (Punt et al., 1994) was significantly reduced on ST thymocytes (including ST thymocytes expressing TCR$\alpha\beta$ transgenes) to levels far below those on normal WT thymocytes (Figures 1C and 1D), suggesting that TCR signal transduction might be defective in ST mice. To assess directly the signaling competence of ST thymocytes, we measured the mobilization of intracellular calcium in freshly isolated $CD4^+CD8^+$ thymocytes from both WT and ST mice. Antibody-induced TCR crosslinking alone or in combination with CD8 failed to mobilize significant amounts of intracellular calcium from any of the mice tested (Figure 2A), as has previously been reported (Nakayama et al., 1990; Wiest et al., 1996). However, antibody induced co-crosslinking of surface TCR and CD4 molecules (TCR + CD4) was highly effective in mobilizing intracellular calcium in $CD4^+CD8^+$ thymocytes from WT normal mice, but failed to mobilize intracellular calcium in ST thymocytes, even ST thymocytes expressing TCR$\alpha\beta$ transgenes (Figure 2A). The failure of ST thymocytes to mobilize intracellular calcium was specific to T lineage cells, since B lymphocytes from ST mice mobilized calcium effectively in response to crosslinking of their antigen receptors (data not shown).

To identify the defective signaling step in ST thymocytes, we examined events in the TCR signaling cascade that were proximal to calcium mobilization. Since activation of phospholipase Cγ (PLCγ) is necessary for hydrolysis of phosphatidylinositol and for intracellular calcium mobilization (June et al., 1990; Secrist et al., 1991; Weiss et al., 1991; Weiss and Littman, 1994), we first asked whether stimulation of ST thymocytes induced phosphorylation of PLCγ (Figure 2B). Even though ST thymocytes expressed normal levels of PLCγ protein, PLCγ phosphorylation was not induced in ST thymocytes by TCR + CD4 co-crosslinking (Figure 2B), suggesting that the signaling defect in ST thymocytes was proximal to PLCγ phosphorylation. Consequently, we next examined tyrosine phosphorylation of the PTK ZAP-70, which is a precondition for TCR-induced calcium mobilization and thus is also likely to be required for TCR-induced PLCγ phosphorylation (Kolanus et al., 1993; Chan et al., 1994a; Bolen, 1995; Gelfand et al., 1995; Wange et al., 1995a; Kong et al., 1996). Interestingly, TCR + CD4 co-crosslinking induced tyrosine phosphorylation of ZAP-70, as detected by anti-phosphotyrosine immunoblotting, in $CD4^+CD8^+$ thymocytes from both normal B6 and

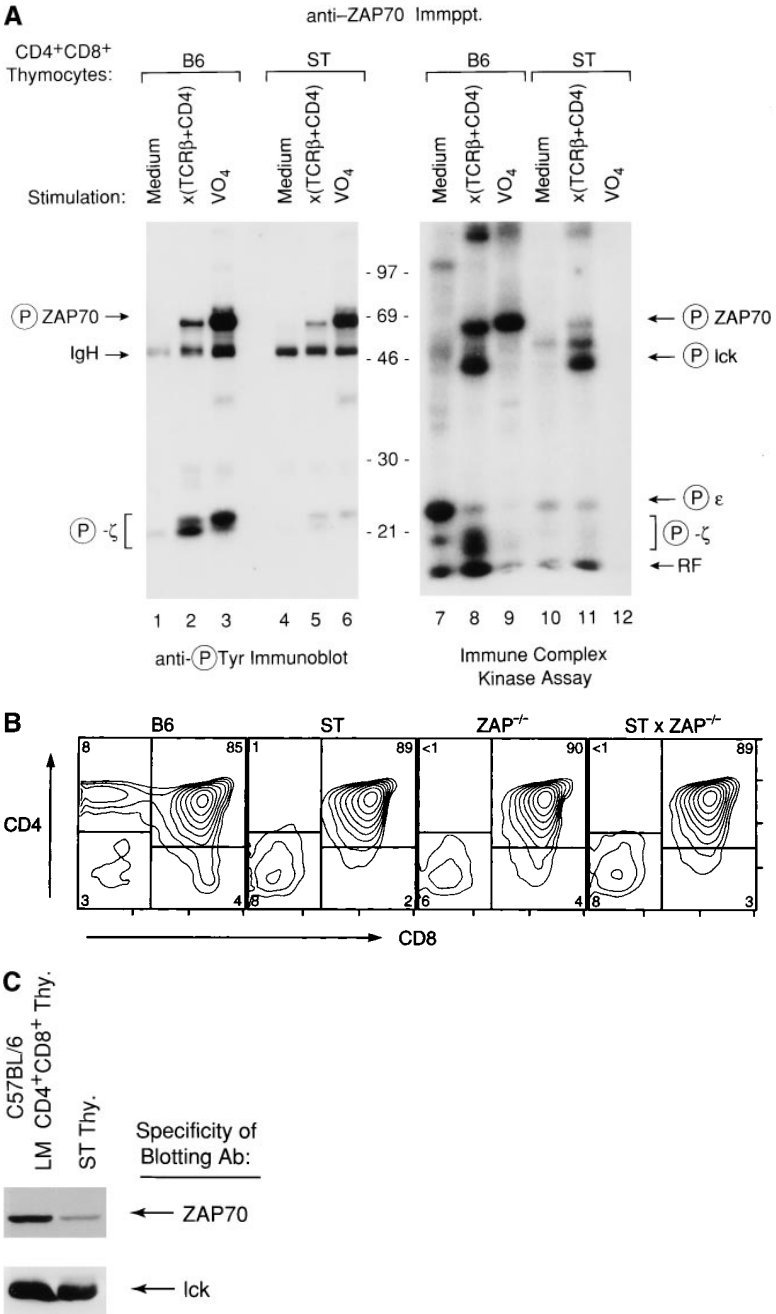

Figure 3. Identification of a Mutation in ZAP-70 as the Cause of the ST Phenotype

(A) Assessment of tyrosine phosphorylation and tyrosine kinase activity in anti-ZAP-70 immunoprecipitates from stimulated $CD4^+CD8^+$ thymocytes. $CD4^+CD8^+$ thymocytes from normal B6 and ST mice were stimulated for 5 min either by TCR + CD4 co-crosslinking or with pervanadate (VO_4), after which anti-ZAP-70 immunoprecipitates were divided into equal halves and evaluated in parallel for tyrosine phosphorylation (left) and in vitro tyrosine kinase activity (right). Radiolabeled bands in the kinase assay result from the incorporation of ^{32}P, revealing the presence of an activated PTK in the anti-ZAP-70 immunoprecipitation. (It might be appreciated that pervanadate oxidizes thiol groups and so can disrupt cysteine-dependent interactions, such as those between CD4 and Lck. Consequently, nonZAP-70–induced background bands that are present in the immune complex kinase assay of anti-ZAP-70 immunoprecipitates from unstimulated cells may be absent in those of pervanadate-stimulated cells.) RF, running front.

(B) The defects in ZAP-$70^{-/-}$ and ST mice are not complementary. Mice homozygous for ablation of their ZAP-70 genes ($ZAP^{-/-}$) were mated with ST mice. Thymi from various mice, including offspring of ST × $ZAP^{-/-}$ matings, were assessed for CD4 versus CD8 expression. Mature $CD4^+$ and $CD8^+$ T cells were lacking in thymi from all (5 of 5) ST × $ZAP^{-/-}$ offspring tested.

(C) ZAP-70 from ST thymocytes is expressed at approximately one fourth of normal levels and displays normal electrophoretic migration. Whole-cell lysates of $CD4^+CD8^+$ thymocytes from normal littermate (LM) and ST mice were electrophoresed and immunoblotted with anti-ZAP-70 antibody and anti-Lck antibody as a loading control. Bound antibodies were visualized by horseradish peroxidase–conjugated protein A and chemiluminescence.

ST mice (Figure 3A, lanes 2 and 5). Thus, the signaling defect in ST mice is distal to ZAP-70 phosphorylation but proximal to PLCγ phosphorylation.

Although ZAP-70 can be phosphorylated at different tyrosine residues, only the phosphorylation of Y493 in its kinase domain is known to be relevant for its activation (Watts et al., 1994; Chan et al., 1995; Wange et al., 1995b; Kong et al., 1996). Therefore, we used TCR + CD4 co-crosslinking to determine whether phosphorylation of ZAP-70 in ST thymocytes actually induced ZAP-70 kinase activity. To do so, anti-ZAP-70 immunoprecipitates from unstimulated and stimulated thymocytes were halved and assayed in parallel by both immune complex kinase assay and anti-phosphotyrosine immunoblot (Figure 3A). In addition to inducing ZAP-70 phosphorylation (Figure 3A, lanes 2 and 5), TCR + CD4 co-crosslinking appeared to induce in vitro PTK activity in anti-ZAP-70 immunoprecipitates from both ST and WT thymocytes (Figure 3A, lanes 8 and 11). However, since the immunoprecipitates also contained detectable Lck molecules (Figure 3A, lanes 8 and 11) that had probably been coprecipitated by the crosslinking anti-CD4 monoclonal antibody (MAb) (Veillette et al., 1988), we could not unequivocally attribute the in vitro PTK activity observed in this experiment to ZAP-70.

To evaluate ZAP-70 kinase activity in the absence of

other kinases, we examined anti-ZAP-70 immunoprecipitates from $CD4^+CD8^+$ thymocytes that had been stimulated with pervanadate, a membrane-permeable phosphatase inhibitor that results in potent activation of intracellular PTKs (O'Shea et al., 1992; Secrist et al., 1993). Pervanadate stimulation, like TCR + CD4 cocrosslinking, induced tyrosine phosphorylation of the ZAP-70 molecules immunoprecipitated from both ST and WT thymocytes (Figure 3A, lanes 3 and 6), but most importantly, the phosphorylated ZAP-70 molecules from ST thymocytes were dramatically devoid of kinase activity (Figure 3A, lanes 9 and 12). Thus, the basis of defective TCR signaling in ST thymocytes appears to be an inability of ZAP-70 phosphorylation to actually induce ZAP-70 kinase activity.

The absence of ZAP-70 kinase activity in ST thymocytes might be the result of a mutation in either the ZAP-70 structural gene itself or in a gene encoding a protein that regulates ZAP-70 kinase activity. To distinguish between these two possibilities, we mated ST mice with ZAP-$70^{-/-}$ mice whose ZAP-70 genes were disrupted by gene targeting (Negishi et al., 1995). As can be seen in Figure 3B, thymocytes from parental ZAP-$70^{-/-}$ and ST mice were identically arrested at the $CD4^+CD8^+$ stage of differentiation. If the ZAP-70 structural gene in ST mice encodes a normal ZAP-70 protein product, then ST $\times$ ZAP-$70^{-/-}$ matings should complement the ST defect and restore thymocyte development in the resultant offspring. Alternatively, if the ZAP-70 structural gene in ST mice encodes a defective ZAP-70 protein product, then ST $\times$ ZAP-$70^{-/-}$ matings will fail to complement and result in defective offspring. In fact, ST $\times$ ZAP-$70^{-/-}$ matings did not complement, since thymocytes from F1 offspring were also arrested at the $CD4^+CD8^+$ stage of differentiation (Figure 3B).

Since ZAP-70 molecules from ST mice were expressed at significant levels and migrated normally on SDS polyacrylamide gel electrophoresis (PAGE) (Figure 3C), the defect in ZAP-70 was likely to be a very limited structural mutation. To identify this mutation, we performed reverse transcriptase polymerase chain reaction (PCR) on RNA derived from ST thymocytes to amplify ZAP-70 mRNA specifically. cDNAs were generated from independently derived RNA samples isolated from either unfractionated thymocytes or from $CD4^+CD8^+$ thymocytes and were subjected to sequence determination. The ST ZAP-70 sequence contained a total of eight nucleotide differences (relative to WT ZAP-70), of which seven were silent and possibly reflect allelic variation. Only one, a C-to-T mutation at nucleotide 1498, altered the amino acid sequence. R at position 464 was changed to C, disrupting the conserved DLAARN motif within the kinase domain of ST ZAP-70 molecules (Figure 4) (Hanks and Quinn, 1991). Thus, the defect that attenuated ZAP-70 kinase activity and arrested thymocyte development in ST mice is a point mutation within the kinase domain of ZAP-70.

Because mutation of the DLAARN motif of ZAP-70 in ST thymocytes resulted both in reduced steady-state levels of ZAP-70 protein and in attenuation of ZAP-70 kinase activity, it was unclear which was responsible for arresting thymocyte development. To distinguish between these possibilities, we mated ZAP-$70^{-/-}$ mice

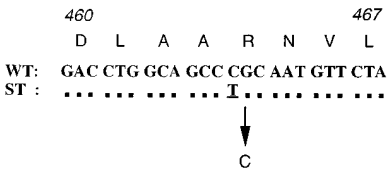

Figure 4. ZAP-70 Molecules from ST Mice Bear a Point Mutation in the Kinase Domain

The amino acid sequence corresponding to residues 460–467 of WT and ST ZAP-70 is shown. The entire ST ZAP-70 nucleotide sequence has been submitted to GenBank (accession number U77667). A total of eight nucleotide differences in the ST ZAP-70 sequence were detected when compared to the ZAP-70 cDNA sequence from normal mice (GenBank accession number UO4379). Of these, only one, a C-to-T change at position 1498 of the ST ZAP-70 sequence, resulted in an amino acid conversion, an R464C mutation in the kinase domain. The seven silent nucleotide differences in the ZAP-70 sequence of ST mice relative to the WT sequence may reflect allelic variations.

with WT B6 mice, reasoning that thymocytes from the resultant B6 $\times$ ZAP$^{-/-}$ progeny would express reduced levels of fully functional ZAP-70 protein (Figure 5A). Indeed, $CD4^+CD8^+$ thymocytes from B6 $\times$ ZAP$^{-/-}$ F1 mice expressed ZAP-70 protein at a level reduced to within 2-fold of that expressed by ST mice (Figure 5A). Furthermore, tyrosine phosphorylation of ZAP-70 molecules associated with TCR complexes on stimulated $CD4^+$ $CD8^+$ thymocytes was virtually identical in B6 $\times$ ZAP$^{-/-}$ and ST mice (Figure 5B). Yet, despite these similarities in ZAP-70 expression and phosphorylation, thymocyte development was completely normal in the B6 $\times$ ZAP$^{-/-}$ mice (Figure 5C). Taken together, these data demonstrate that the arrest in thymocyte development in ST mice does not result from reduced levels of ZAP-70 protein but rather from abrogation of ZAP-70 kinase activity.

Discussion

The present study demonstrates that an R-to-C mutation in the conserved DLAARN motif abrogates ZAP-70 kinase activity, resulting in defective TCR signaling and complete arrest of thymocyte development at the $CD4^+CD8^+$ stage of differentiation. In fact, while DLAARN is conserved among all PTKs, this is the first demonstration that a mutation of the R residue affects kinase activity in any PTK molecule.

Mutations of ZAP-70 have not previously been described in mice but have been found in humans with a novel form of severe combined immunodeficiency disease (SCID) (Arpaia et al., 1994; Chan et al., 1994b; Elder et al., 1994). The immunodeficiency in these SCID patients is characterized by the absence of peripheral $CD8^+$ T cells and the presence of nonfunctional $CD4^+$ T cells. Interestingly, thymocytes in these patients are able to mobilize intracellular calcium in response to TCR stimulation (Qian et al., 1996). In contrast, ST mice are virtually devoid of peripheral T cells, and TCR signaling in ST thymocytes has been completely abrogated. This difference in signaling ability may explain why humans with defects in ZAP-70 produce mature T cells, whereas ST mice do not. All of the mutations of ZAP-70 that have

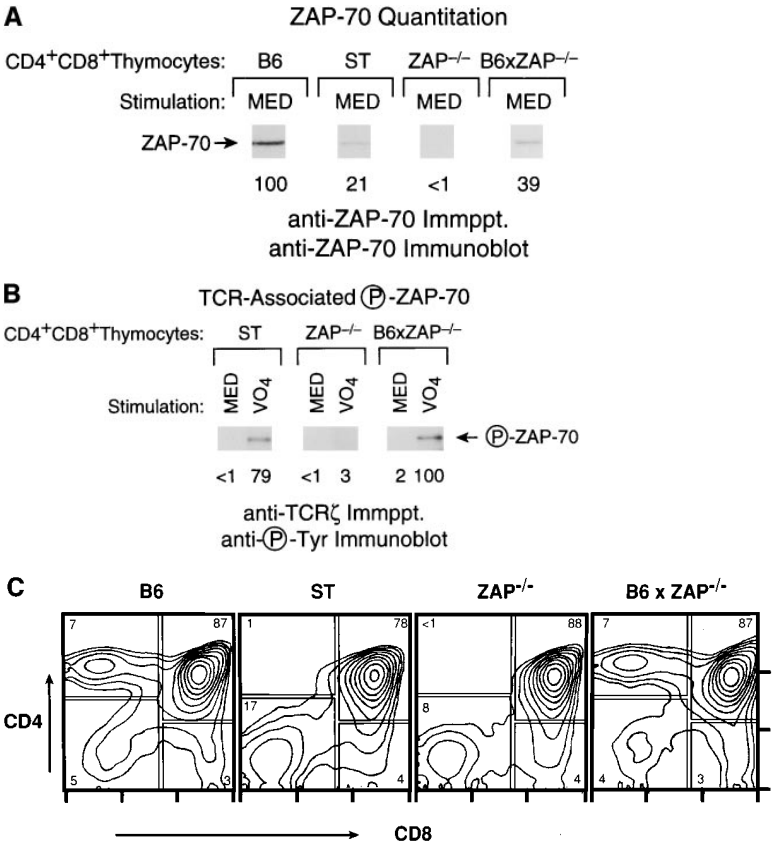

Figure 5. Comparative Analysis of ZAP-70 Expression and Function in ST versus B6 × ZAP$^{-/-}$ F1 Thymocytes

(A) ZAP-70 expression levels in ST CD4$^+$CD8$^+$ thymocytes are within 2-fold of that expressed by CD4$^+$CD8$^+$ thymocytes from B6 × ZAP$^{-/-}$ mice. ZAP-70 molecules were immunoprecipitated from detergent extracts of CD4$^+$CD8$^+$ thymocytes isolated from the indicated strains of mice. Thymocytes from age- and sex-matched animals were compared, and ZAP-70 protein content of the immunoprecipitations was analyzed by immunoblotting. MED, medium.

(B) Stimulation of ST thymocytes induces tyrosine phosphorylation of TCR-associated ZAP-70 molecules that is equivalent to that seen in thymocytes from B6 × ZAP$^{-/-}$ mice. CD4$^+$CD8$^+$ thymocytes from ST, ZAP$^{-/-}$, and B6 × ZAP$^{-/-}$ mice were stimulated with pervanadate and extracted with detergent and the lysates immunoprecipitated with anti-TCRζ antibody. The resultant immunoprecipitations were analyzed by anti-phosphotyrosine immunoblotting.

(C) Thymocyte development is not disrupted in B6 × ZAP$^{-/-}$ animals. Thymi from the indicated mice were assessed for CD4 versus CD8. Notably, mature CD4$^+$ and CD8$^+$ thymocytes were present in B6 × ZAP$^{-/-}$ but not ST mice.

been reported to cause SCID in humans cluster in the kinase domain between amino acids 504 and 542 (Figure 6), and all result in instability of ZAP-70 protein, virtually eliminating its expression in these patients. Consequently, the T cell defects in these patients may simply reflect the loss of ZAP-70 protein. In contrast, the R464C ZAP-70 mutation in ST mice does not eliminate ZAP-70 expression; rather, the ZAP-70 molecules present in ST thymocytes cannot be activated. In fact, abrogation of ZAP-70 kinase activity by the novel, spontaneously arising R464C ST mutation has a potential structural basis, provided by analogy with the crystal structure of the human insulin receptor PTK domain. This crystallographic analysis predicts that the R residue of the DLAARN motif, which comprises the catalytic loop of the kinase domain, might influence the phosphotransferase active site of PTKs both by neutralizing charges at the phosphotransfer site and by hydrogen boding with the catalytic base (Hubbard et al., 1994).

Importantly, since ST mice express a catalytically inactive form of ZAP-70, the present study represents the first demonstration that ZAP-70 PTK activity is necessary for the biological role of ZAP-70 in vivo in thymocyte development. ZAP-70 kinase activity is necessary for early TCR signaling events such as PLCγ phosphorylation and calcium mobilization as well as for very distal events such as the cell fate decisions involved in positive selection. These findings support and significantly extend the recent demonstration that ZAP-70 kinase activity was necessary for TCR signaling in an in vitro cell line (Qian et al., 1996). There are two mechanisms whereby ZAP-70 might mediate TCR signaling in thymocyte development: ZAP-70 might phosphorylate downstream cytosolic signaling molecules that are part of the TCR signaling cascade (Wardenburg et al., 1996); alternatively, ZAP-70, when phosphorylated by p56^{lck}, might serve as a molecular scaffold or linker protein that attracts cytosolic signaling molecules to the TCR complex. The requirement for ZAP-70 enzymatic activity demonstrated in this report is consistent with ZAP-70 phosphorylation of downstream targets and excludes the possibility that exogenous phosphorylation of ZAP-70 is sufficient to form a molecular scaffold for TCR signaling in thymocyte development. Rather, if ZAP-70 does have a significant role as a molecular scaffold in TCR signaling in vivo, autophosphorylation of molecular docking sites by its own intrinsic kinase activity is required (Neumeister et al., 1995).

Finally, it should be noted that we originally detected the ST mutation in a colony of mice that had been derived from embryonic stem cells whose CD28 gene had been disrupted by gene targeting. Since the ST mutation is entirely independent of CD28 expression, its occurrence in a CD28 knockout mouse colony was simply fortuitous. Since the ST mutation is a point mutation, it cannot have been a direct consequence of the gene targeting procedure to which the original embryonic stem cells were subjected, a conclusion that is also consistent with our failure to detect the gene targeting vector in genomic DNA from ST mice. However, origination of the ST mutation in the CD28$^{+/-}$ mouse colony indicates that the mutation occurred either in the

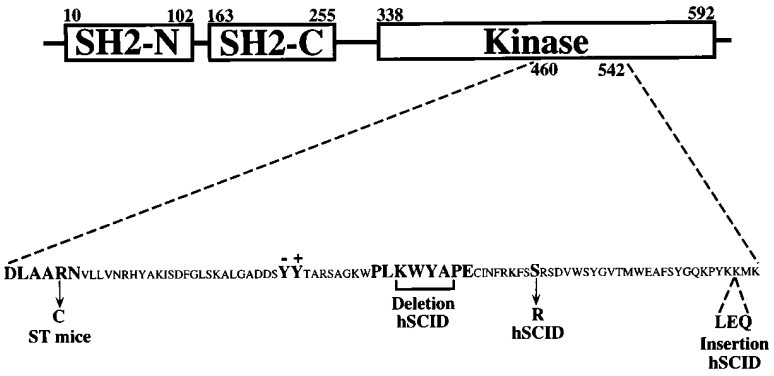

Figure 6. ZAP-70 Mutations Producing Immunodeficiency in Mice and Humans

The position and type of known mutations within ZAP-70 kinase domain that produce immunodeficiency are indicated: (1) deletion hSCID, a 13 bp deletion spanning nucleotides 1719–1731, observed in human immunodeficiency; (2) R hSCID, a point mutation at nucleotide 1763 that changes serine to arginine at position 518 in human immunodeficiency; and (3) insertion hSCID, a 9 bp insertion at nucleotide 1832 that introduces the tripeptide leucine–glutamic acid–glutamine (LEQ) at position 542 (Arpaia et al., 1994; Chan et al., 1994b; Elder et al., 1994). + indicates the increased kinase activity upon phosphorylation of Y493; − indicates the observed inhibitory effect of Y492 on kinase activity (Watts et al., 1994; Chan et al., 1995; Wange et al., 1995).

$CD28^{+/-}$ mouse colony itself or in the embryonic stem cells from which these mice were originally derived. In either case, its occurrence serves as a demonstration that dramatic phenotypes can arise as a result of spontaneous mutations in embryonic stem cell–derived mice that may be unrelated to the identity of the originally targeted gene of interest.

Experimental Procedures

Mice

Young adult C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, MD). The following transgenic and gene-targeted mice were used in this study: (1) DO11.10 TCR transgenic mice obtained from Dr. D. Y. Loh (Nippon Roche Research Center) (Murphy et al., 1990); (2) $CD28^{-/-}$ mice obtained from Dr. Tak Mak (Toronto University) (Shahinian et al., 1993); and (3) $ZAP\text{-}70^{-/-}$ mice generated as described (Negishi et al., 1995).

Intercross Analysis

Mice with the ST phenotype arose during intercrossing $CD28^{-/-}$ with B6 mice. The possibility that the ST mutation was a defect in TCRα or β subunits was assessed by intercrossing ST mice with mice expressing the transgenic TCR$\alpha\beta$ subunits of the DO11.10 TCR. F1 progeny from this mating were intercrossed. Thirty F2 animals from these matings were examined. Of the 30, approximately 75% bore the DO11.10 transgenic TCR as indicated by PCR analysis on tail DNA using the following primers: Vα13.1 5′-CAGGAGGGATC CAGTGCCAGC-3′ and Jα 5′-TGGCTCTACAGTGAGTTT GGT 3′. Of the transgenic F2 offspring, 25% had the ST phenotype, as evidenced by the absence of peripheral blood T cells. This is the expected frequency for an autosomal recessive trait among F2 progeny in such a breeding regimen.

Antibodies

The following fluor-conjugated MAbs purchased from Pharmingen (SanDiego, CA) were used in flow cytometry: fluoresceinated anti-TCRβ (H57–597) (Kubo et al., 1989); fluoresceinated anti-CD4 (RM4.5); phycoerythrin-anti-CD8 (53–6.7) (Ledbetter et al., 1980); fluoresceinated anti-B220 (RA3–6B2) (Asensi et al., 1989); phycoerythrin-anti-Thy1.2 (30-H12) (Ledbetter et al., 1980); and phycoerythrin–anti-CD5 (53–7.3) (Ledbetter et al., 1980). The following antibodies were used in signaling experiments: anti-CD4 (GK1.5) (Dialynas et al., 1983); anti-CD8 (2.43) (Dialynas et al., 1983); anti-TCRβ (H57–597); anti-phosphotyrosine (4G10) (UBI, Lake Placid, NY); rabbit anti-ZAP-70 (Burkhardt et al., 1994); rabbit anti-Lck (serum 688) (Samelson et al., 1990); and anti-PLCγ (UBI).

Cell Preparation and Flow Cytometry

Single cell suspensions were produced from freshly explanted thymus, spleen, and lymph node tissue and then analyzed by flow cytometry as described (Nakayama et al., 1989). Thymic explants from which $CD4^+CD8^+$ thymocytes were to be isolated were strictly kept at 4°C during isolation and purification. $CD4^+CD8^+$ thymocytes were isolated by panning on anti-CD8 coated plates (Nakayama et al., 1989) and were consistently greater than 96% $CD4^+CD8^+$.

Surface Biotin Labeling and Two-Dimensional Electrophoresis

$CD4^+CD8^+$ thymocytes from DO11.10 TCR transgenic WT and ST mice were surface-labeled with biotin, extracted with lysis buffer containing 1% digitonin (Wako, Kyoto, Japan), and immunoprecipitated with anti-TCRβ (H57–597) MAb adsorbed to protein A–Sepharose beads as described (Wiest et al., 1995). The resultant immune complexes were resolved on two-dimensional SDS-PAGE gels and visualized using horseradish peroxidase–conjugated streptavidin and chemiluminescence as described (Wiest et al., 1995).

Calcium Mobilization

$CD4^+CD8^+$ thymocytes were strictly maintained at 4°C until they were loaded at 10^7/ml in 2% fetal calf serum/RPMI with 1 μg/ml indo-1 (Molecular Probes, Eugene, OR) for 30 min at 31°C. The cells were then stained for 20 min at 4°C with 1 μg each of the indicated biotinylated MAbs: anti-TCRβ (H57–597); anti-CD4 (GK1.5); and anti-CD8 (2.43). After washing with 0.5% bovine serum albumin/Hank's balanced salt solution at 4°C, the cells were warmed to 37°C and immediately loaded into an Epics Elite cytometer (Coulter, Hialeah, FL). Calcium mobilization was analyzed following avidin crosslinking (4 μg/ml) by monitoring the fluorescence emission ratio at 395 nM:525 nM after excitation of indo-1 at 325 nM with a helium-cadmium laser. Data were analyzed using the Multitime program (Phoenix Flow Systems, San Diego, CA).

Stimulation

Freshly isolated $CD4^+CD8^+$ thymocytes (50–80 $\times$ 10^6 per sample) were pelleted and resuspended at 10^7/ml in ice cold RPMI containing 1 mM Na_3VO_4 and 10 μg/ml each of the biotinylated MAbs anti-CD4 and anti-TCRβ. Cells were stained for 10 min at 4°C and then pelleted and resuspended in 1 ml of 20 μg/ml streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL) that was prewarmed to 37°C. After a 5 min incubation at 37°C, the cells were immediately lysed in buffer containing 1% Triton X-100 detergent and phosphatase inhibitors (1 mM Na_3VO_4 and 2 mM EDTA) and immunoprecipitated with the indicated antibody, either anti-PLCγ or anti-ZAP-70. $CD4^+CD8^+$ thymocytes to be stimulated with pervanadate were resuspended at 10^7/ml in a 37°C solution containing 0.3 mM H_2O_2 and 0.1 mM Na_3VO_4 and incubated for 10 min at 37°C as described (Wiest et al., 1996). Immune complexes were then analyzed either by anti-phosphotyrosine immunoblotting or by immune complex kinase assay as indicated. Immune complexes to be analyzed by anti-phosphotyrosine immunoblots were eluted in SDS-sample buffer, resolved by SDS-PAGE, and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA) as described (Wiest et al., 1993). Tyrosine phosphorylated proteins were identified by probing

blots with anti-phosphotyrosine MAb (1 μg/ml) and developing them with horseradish peroxidase–conjugated protein-A (Kirkegaard Perry Laboratories, Gaithersburg, MD) diluted 1:2500 in phosphate-buffered saline containing 0.4% Tween-20 followed by chemiluminescence biotin (SuperSignal, Pierce Chemical, Rockford, IL). PLCγ and Lck were identified by probing blots with anti-PLCγ and anti-Lck as described (Wiest et al., 1996). Immune complexes to be analyzed by kinase assay were incubated at ambient temperature for 3 min in kinase buffer containing 15 μCi/sample of γ^{32}P-ATP (ICN Biomedicals), after which the immune complexes were resolved by SDS-PAGE and visualized by autoradiography (Wiest et al., 1993). Radiolabeled proteins in the immune complex kinase assay reflect transfer of ^{32}P by an activated PTK molecule present in the anti-ZAP-70 immunoprecipitation.

Sequence Analysis of ZAP-70 from ST Mice

Total RNA was derived from ST thymocytes using the guanidine isothiocyanate method as described (Sambrook et al., 1989). ST ZAP-70 cDNA was synthesized using rTth DNA polymerase, XL (Perkin-Elmer, Norwalk, CT), and antisense primers 5′-CTCAGCCACAT GCAGCCTCGGCCACCTGTTC-3′ or 5′-CCATGGCCACCTGGTGCA GCAGTTCAGC-3′, from which the amino- and carboxy-terminal regions of the ST ZAP-70 cDNA were PCR-amplified using sense primers 5′-ATGCCCGATCCCGCGGCGCACCTGCCATTCTT-3′ and 5′-GCTGAACTGCTGCACCAGGTGGCCATGG-3′, respectively. Amplified fragments were incubated with Taq polymerase for an additional 10 min at 72°C and subcloned in the pCRII cloning vector (Invitrogen, San Diego, CA). Plasmid minipreps containing the mutant ZAP-70 cDNA were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer), a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA), and an ABI 373 DNA Sequencer (Perkin Elmer). The resultant cDNAs were then subjected to DNA sequence determination; overlapping cDNAs were sequenced in both directions. As a control, WT ZAP-70 cDNA (provided by Dr. Andrey Shaw, GenBank accession number UO4379) was sequenced in parallel.

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References

Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C.M. (1994). Defective T cell receptor signaling and $CD8^+$ thymic selection in humans lacking Zap-70. Cell *76*, 947–958.

Asensi, V., Kimeno, K., Kawamura, I., and Sakumoto, M. (1989). Treatment of autoimmune MRL/lpr mice with anti-B220 monoclonal antibody reduces the level of anti-DNA antibodies and lymphadenopathies. Immunology *68*, 204–208.

Bolen, J.B. (1995). Protein tyrosine kinases in the initiation of antigen receptor signaling. Curr. Opin. Immunol. *7*, 306–311.

Burkhardt, A., Stealey, B., Rowley, R.B., Mahajan, S., Prendergast, M., Fargnoli, J., and Bolen, J.B. (1994). Temporal regulation of nontransmembrane protein tyrosine kinase enzyme activity following T cell antien receptor engagement. J. Biol. Chem. *269*, 23642–23647.

Chan, A.C., Irving, B.A., Fraser, J.D., and Weiss, A. (1991). The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc. Natl. Acad. Sci. USA *88*, 9166–9170.

Chan, A.C., Iwashima, M., Turck, C.W., and Weiss, A. (1992). ZAP-70: a 70 kD protein-tyrosine kinase that associates with the TCR zeta chain. Cell *71*, 649–662.

Chan, A.C., Desai, D.M., and Weiss, A. (1994a). The role of protein-tyrosine kinases and protein-tyrosine phosphatases in T cell antigen receptor signal transduction. Annu. Rev. Immunol. *12*, 555–592.

Chan, A.C., Kadlecek, T.A., Elder, M.E., Filipovich, A.H., Kuo, W.L., Iwashima, M., Parslow, T.G., and Weiss, A. (1994b). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. Science *264*, 1599–1601.

Chan, A.C., Dalton, M., Johnson, R., Kong, G., Wang, T., Thoma, R., and Kurasaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO J. *14*, 2499–2508.

Dialynas, D.P., Wilde, D.B., Marrack, P., Peirres, A., Wall, K.A., Havran, W., Otten, G., Loken, M.R., Pierres, M., Kappler, J., et al. (1983). Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. Immunol. Rev. *74*, 29–56.

Elder, M.E., Lin, D., Clever, J., Chan, A.C., Hope, T.J., Weiss, A., and Parslow, T.G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. Science *264*, 1596–1599.

Gelfand, E.W., Weinberg, K., Mazer, B.D., Kadlecek, T.A., and Weiss, A. (1995). Absence of ZAP-70 prevents signaling through the antigen receptor on peripheral blood T cells but not on thymocytes. J. Exp. Med. *182*, 1057–1066.

Hanks, S.K., and Quinn, A.M. (1991). Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Meth. Enzymol. *200*, 38–62.

Hashimoto, K., Sohn, S.J., Levin, S.D., Tada, T., Perlmutter, R.M., and Nakayama, T. (1996). Requirement for p56(lck) tyrosine kinase activation in T cell receptor-mediated thymic selection. J. Exp. Med. *184*, 931–943.

Hubbard, S.R., Wei, L., Ellis, L., and Hendrickson, W.A. (1994). Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature *372*, 746–754.

June, C.H., Fletcher, M.C., Ledbetter, J.A., Schieven, G.L., Siegel, J.N., Phillips, A.F., and Samelson, L.E. (1990). Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc. Natl. Acad. Sci. USA *87*, 7722–7726.

Kolanus, W., Romeo, C., and Seed, B. (1993). T cell activation by clustered tyrosine kinases. Cell *74*, 171–183.

Kong, G., Dalton, M., Wardenburg, J.B., Straus, D., Kurosaki, T., and Chan, A.C. (1996). Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. Mol. Cell Biol. *16*, 5026–5035.

Kubo, R.T., Born, W., Kappler, J.W., Marrack, P., and Pigeon, M. (1989). Characterization of a monoclonal antibody which detects all murine αβ T cell receptors. J. Immunol. *142*, 2736–2742.

Ledbetter, J.A., Rouse, R.V., Micklem, H.S., and Herzenberg, L.A. (1980). T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. Two-parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. J. Exp. Med. *152*, 280–295.

Littman, D.R., Davis, C.B., Killeen, N., and Xu, H. (1994). Signal transduction during T cell development. Adv. Exp. Med. Biol. *365*, 63–71.

Murphy, K.M., Heimberger, A.B., and Loh, D.Y. (1990). Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+$ TCR^{lo} thymocytes in vivo. Science *250*, 1720–1723.

Nakayama, T., June, C.H., Munitz, T.I., Sheard, M., McCarthy, S.A., Sharrow, S.O., Samelson, L.E., and Singer, A. (1990). Inhibition of T cell receptor expression and function in immature $CD4^+CD8^+$ cells by CD4. Science *249*, 1558–1561.

Nakayama, T., Singer, A., Hsi, E.D., and Samelson, L.E. (1989). Intrathymic signaling in immature $CD4^+CD8^+$ thymocytes results in tyrosine phosphorylation of the T cell receptor ζ chain. Nature *341*, 651–654.

Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C., and Loh, D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435–438.

Neumeister, E.N., Zhu, Y., Richard, S., Terhorst, C., Chan, A.C., and Shaw, A.S. (1995). Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. Mol. Cell Biol. *15*, 3171–3178.

O'Shea, J.J., McVicar, D.W., Bailey, T.L., Burns, C., and Smyth, M.J. (1992). Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA *89*, 10306–10310.

Punt, J.A., Osborne, B.A., Takahama, Y., Sharrow, S.O., and Singer, A. (1994). Negative selection of $CD4^+CD8^+$ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. J. Exp. Med. *179*, 709–713.

Qian, D., Mollenauer, M.N., and Weiss, A. (1996). Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. J. Exp. Med. *183*, 611–620.

Robey, E., and Fowlkes, B.J. (1994). Selective events in T cell development. Annu. Rev. Immunol. *12*, 675–705.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Plainview, NY: Cold Spring Harbor Laboratory Press).

Samelson, L.E., Phillips, A.F., Luong, E.T., and Klausner, R.D. (1990). Association of fyn protein-tyrosine kinase with the T-cell antigen receptor. Proc. Natl. Acad. Sci. USA *87*, 4358–4362.

Secrist, J.P., Karnitz, L., and Abraham, R.T. (1991). T-cell antigen receptor ligation induces tyrosine phosphorylation of phospholipase Cγ1. J. Biol. Chem. *266*, 12135–12139.

Secrist, J.P., Burns, L.A., Karnitz, L., Koretzky, G.A., and Abraham, R.T. (1993). Stimulatory effects of the protein tyrosine phosphatase inhibitor, pervanadate, on T-cell activation events. J. Biol. Chem. *268*, 5886–5893.

Shahinian, A., Pfeffer, K., Lee, K.P., Kundig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B., and Mak, T.W. (1993). Differential T cell co-stimulatory requirements in CD28-deficient mice. Science *261*, 609–612.

Veillette, A., Bookman, M.A., Horak, E.M., and Bolen, J.B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell *55*, 301–308.

Wange, R.L., Isakov, N., Burke, T.R., Otaka, A., Roller, P.P., Watts, J.D., Aebersold, R., and Samelson, L.E. (1995a). F2(Pmp)2-TAM zeta 3, a novel competative inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early T cell signaling. J. Biol. Chem. *270*, 944–948.

Wange, R.L., Guitian, R., Isakov, N., Watts, J.D., Aebersold, R., and Samelson, L.E. (1995b). Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. J. Biol. Chem. *270*, 18730–18733.

Wardenburg, J.B., Fu, C., Jackman, J.K., Flotow, H., Wilkinson, S.E., Williams, D.H., Johnson, R., Kong, G., Chan, A.C., and Findell, P.R. (1996). Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J. Biol. Chem. *271*, 19641–19644.

Watts, J.D., Affolter, M., Krebs, D.L., Wange, R.L., Samelson, L.E., and Aebersold, R. (1994). Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. J. Biol. Chem. *269*, 29520–29529.

Weiss, A., Koretzky, G., Schatzman, R., and Kadlecek, T. (1991). Stimulation of the T cell antigen receptor induces tyrosine phosphorylation of phospholipase C-γ1. Proc. Natl. Acad. Sci. USA *88*, 5484–5488.

Weiss, A., and Littman, D. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Wiest, D.L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R.D., Glimcher, L.H., Samelson, L.E., and Singer, A. (1993). Regulation of T cell receptor expression in immature $CD4^+CD8^+$ thymocytes by $p56^{lck}$ tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J. Exp. Med. *178*, 1701–1712.

Wiest, D.L., Burgess, W., McKean, D., Kearse, K.P., and Singer, A. (1995). The molecular chaperone calnexin is expressed on the surface of immature thymocytes in association with clonotype-independent CD3 complexes. EMBO J. *14*, 3425–3433.

Wiest, D.L., Ashe, J.M., Abe, R., Bolen, J.B., and Singer, A. (1996). TCR activation of ZAP70 is impaired in $CD4^+CD8^+$ thymocytes as a consequence of intrathymic interactions that diminish available $p56^{lck}$. Immunity *4*, 495–504.